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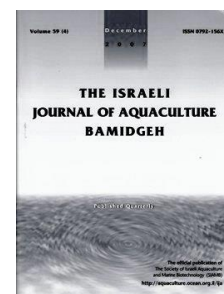
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Application Frequency of Dietary *Vibrio harveyi* Lipopolysaccharide (LPS) on Growth and White Spot Syndrome Virus Resistance of Post Larvae *Penaeus monodon*

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Key words: *Vibrio harveyi*, lipopolysaccharide, *Penaeus monodon*, white spot syndrome virus, total hemocyte count, phenoloxidase activity, respiratory burst activity

Abstract

This study evaluated the effects of dietary *Vibrio harveyi* lipopolysaccharide (LPS) as an immunostimulant fed at different frequencies for improving growth performance, immune response, and disease resistance of post larvae *Penaeus monodon* against white spot syndrome virus (WSSV). In an 8 week feeding trial, shrimp were fed a treatment diet containing *Vibrio harveyi* LPS at a predetermined dose of 50 mg/kg diet daily or every 2, 5, and 7 days. A basal diet was given to the treatment groups when the shrimp were not fed the dietary immunostimulant. Results showed 90-96% survival of the test animals, and weight gain (WG) and specific growth rate (SGR) were significantly enhanced in shrimp fed LPS every 2 days. No significant differences were observed in the feed conversion ratio (FCR) of any of the test groups. Following WSSV challenge, the groups receiving LPS-supplemented diet exhibited increased disease resistance compared to the control group. Shrimp fed with LPS every 2 days exhibited significantly higher survival rate (53%) than the other treatments. The same group consistently showed significantly higher values in all immune indices measured including total hemocyte count (THC), phenoloxidase (PO) activity, and respiratory burst activity (RBA). This study suggests that dietary administration of *Vibrio harveyi* LPS at 50 mg/kg concentration fed every 2 days is optimum to enhance growth, immune response, and protection against WSSV in post larval *P. monodon*.

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Introduction

Penaeus monodon, also known as black tiger shrimp, is a major aquaculture product in the Philippines and Southeast Asia. The decline of this industry in the late 90's was attributed to the outbreak of bacterial vibriosis caused by various species of *Vibrio* (Lavilla-Pitogo et al., 1996). Overall shrimp production volume has not improved and viral diseases continue to plague the region. Among these, white spot syndrome virus (WSSV), is considered to be the most detrimental, affecting almost all phases of shrimp farming, including hatcheries, nurseries, and grow-out ponds around Asia (Chou et al., 2005).

WSSV which is an envelope, non-occluded, and rod-shaped baculovirus (Wang et al., 1995), manifested by white spots in the exoskeleton of shrimp and characterized by occurrence of sudden mass mortality, was first reported in Thailand in 1994 (Takahashi et al., 1994). The disease has since spread throughout the Asian region.

Global economic losses caused by WSSV have spurred research into both prophylactic and curative measures. The use of chemical agents such as antibiotics and disinfectants in culture ponds for disease control is not economically practical and the threat of environmental contamination by them is unavoidable. In crustaceans and other invertebrates, their physical barriers serve to confine invading pathogens (Soderhall, 1982). Despite lacking the adaptive immunity or immunological memory of vertebrates, crustaceans possess efficient defense mechanisms. Certain physiological factors are significantly involved in the immunity of these animals (Vasquez et al., 1999; Vasquez et al., 2009). There are many studies on immunostimulants which are "eco-prophylactic agents. These enhance the innate (non-specific) immune defenses of fish and crustaceans providing resistance against potentially invasive and disease-causing organisms, and promoting better growth and performance" (Citarasu et al., 2006). Most common immunostimulants are glucans derived from yeast, lipopolysaccharides, and peptidoglycan from bacteria, inactivated bacteria, viruses, and dsRNA (Yodmuang et al., 2006). Despite favorable reports on the effects of immunostimulants on fish and crustaceans, several concerns have been raised regarding dose, timing, duration, and frequency of administration. Studies on frequency are lacking. Overdoses and prolonged administration of immunostimulants may reduce their efficacy (Sakai, 1999) and may even lead to immunosuppression that eventually provides less protection from infection of potent pathogens (Sajeevan et al., 2009).

Vibrios are some of the most important Gram-negative groups of bacteria that thrive in brackish and marine water environments. Their outer membrane surface is composed mainly of lipopolysaccharide (LPS), a strong stimulator of immune defense in animals (Bayston and Cohen, 1990; Kim et al., 2000; Alexander and Rietschel, 2001). LPS is a macromolecule with a molecular mass of 10-20 kDa made up of three structural components: a hydrophobic lipid section (lipid A) responsible for the toxic properties of the compound, a hydrophilic core polysaccharide chain, and a repeating hydrophilic O-antigen side chain specific to the bacterial species. The endotoxicity of LPS is facilitated by the secretion of inflammatory factors from immune responsive cells such as macrophages. Recognition of this endotoxin generates a complex cascade of intracellular signals (Bayston and Cohen, 1990).

Study of the biological effects of bacterial LPS on aquatic animals has grown and it is a commonly used feed additive in fish, salmonids (Skalli et al. 2013; Selvaraj et al. 2006) grass carp (Sun et al., 2012) and others, and is a potent immunostimulant in crustaceans (Iwanaga et al., 1994; Takahashi et al., 2000; Wang et al., 2005). It has been reported that this compound is involved in the activation of various immune responses in shrimp such as phenoloxidase (PO) activity, phagocytosis, production of superoxide anion, and expression of antiviral genes (Little et al., 2005).

To the best of our knowledge, no studies have been reported on the effects of *V. harveyi* LPS on black tiger shrimp against WSSV infection. This study was conducted to determine the optimum application frequency of this LPS and its effects on the resistance and immune responses of *P. monodon* post larvae to WSSV. In a preliminary experiment, a dose of 50 mg LPS/kg diet enhanced survival of *P. monodon* post larvae against WSSV by 72% (Genio et al., 2014). With this predetermined dose, LPS was tested at varying feeding frequencies and its immunological indices were investigated.

Materials and Methods

Experimental animals and design. *P. monodon* larvae (0.001 g) were obtained from a commercial hatchery in Tigbauan, Iloilo, Philippines and subjected to microscopic examination for tissue necrosis, PCR analyses for monodon baculovirus (MBV), and WSSV presence to ensure healthy, disease-free stock. The subjects were acclimatized, and fed a commercial diet for one week in moderately aerated water in a 500 L fiberglass tank at the Multi-Species IA Hatchery of University of the Philippines Visayas, Miagao, Iloilo. Shrimp were then transferred to a 5-ton canvas tank and reared for one week to a body weight of 0.02 g. They were then randomly distributed to fifteen 50 L aquaria in a recirculating system and stocked at a density of 25 shrimp per aquarium which were then divided in 4 treatment groups with three replicates fed with LPS-supplemented diets daily, after 2, 5, 7 days, respectively, and a control diet with no immunostimulant. The trial was a completely randomized design. A basal diet with no immunostimulant was given to the treatment groups during the periods when no treated food was administered. Salinity was maintained at 18-20 ppt, and temperature at 27-29°C throughout the experiment.

Treatment groups were fed the test diets at a rate of 15% of the shrimp biomass and were adjusted to 10% after four weeks. Feed was administered three times a day at 08:00, 12:00, and 16:00 hours for a period of 8 weeks. Sampling was carried out every two weeks to measure growth. Prior to the morning feed, excess feed and animal waste was siphoned out, and 30% of water was changed. Water quality was monitored and maintained at an optimum level throughout the experiment. Ammonia and nitrite were monitored weekly.

Bacterial culture. The luminous pathogenic *V. harveyi* PN 9801 isolate (de la Peña *et al.*, 2001) was obtained from the bacterial collection of Southeast Asian Fisheries Development Center (SEAFDEC) in Tigbauan, Iloilo. The isolate was maintained in 1/3 strength nutrient agar slants with 1.5% NaCl at room temperature. The bacteria were inoculated in a nutrient agar medium containing 1.5% NaCl by streaking, and subcultured into fresh medium after 18-20 hours of incubation. Remaining cells were collected by scraping the agar surface using a sterile spatula. Harvested cells were stored at -80 °C until use. Subsequent cultures were carried out using 15 large agar plates for two weeks until the desired amount of bacterial pellets was obtained.

Extraction of LPS. Crude LPS was extracted from *V. harveyi* by the hot phenol-water extraction method (Rezania *et al.*, 2011) with slight modifications. Bacterial pellets were collected by centrifugation at 5,000 x *g* for 20 min. The pellets were washed twice in phosphate buffered saline (PBS) containing 0.15 mM CaCl₂ and 0.5 mM MgCl₂, resuspended in 10 ml PBS, and sonicated for 10 min on ice. Following incubation, an equal volume of 90% hot phenol (65-70°C) was added to the samples, stirred vigorously for 15 min using a magnetic stirrer, and cooled on ice. The mixture was transferred to polypropylene tubes and centrifuged at 5000 x *g* for 20 min. The phenol-saturated water layer containing LPS extract supernatant was removed and transferred to new polypropylene tubes. The interphase and bottom layers were re-extracted with 90% hot phenol to maximize yield.

Sodium acetate at 0.5 M final concentration and 10 volumes of 95% ethanol were added to the extracts and stored at -20°C overnight to precipitate LPS. Following precipitation, LPS extracts were collected by centrifugation at 2000 x *g* at 4°C for 10 min, resuspended in distilled water, and subjected to dialysis for three days to remove phenol residue. The final LPS product was collected and stored at -80 °C until use.

Preparation of test diets. The basal diet was based on the Deshimaru *et al.* (1984) formulation with slight modifications. This has been shown to promote optimum growth in *P. monodon* (Table 1). The experimental bacterial LPS was incorporated into the basal diet at a rate of 50 mg/kg diet on a w/w basis. Cellulose served as filler and LignoBond served as binder and pellet stabilizer. These compounds have no known effects on shrimp since they are indigestible.

The wet and dry ingredients were mixed separately. LPS was dissolved in oil and diethyl ether was added to dilute the viscous lecithin for proper distribution in the feed.

To ensure that a very small concentration of LPS was well-distributed into the diet, the wet ingredients mixture was sonicated until the liquid exhibited a notable change in color. The sonicated mixture was then added to the previously mixed dry ingredients. All ingredients were thoroughly mixed to a dough-like consistency and then a 2-mm die pelletizer was used to produce spaghetti-like pellets which were then oven-dried overnight at 60° C. Dried feeds were stored at -20° C until use.

Table 1. Basal Feed Composition

<i>Ingredients</i>	<i>Amount (g/100g)</i>
Acetes meal	35.0
Peruvian fish meal	25.0
Gelatin	15.0
Wheat flour	15.8
Cod liver oil	3.5
LignoBond	1.5
Mineral mix	1.5
Vitamin mix	1.5
Cellulose	0.195
Test immunostimulant	0.005
Lecithin	1.0
Total	100.0

Growth performance. Specific growth rate (SGR), % weight gain (WG), feed conversion ratio (FCR), and protein efficiency ratio (PER) of shrimp were measured as indicators of growth performance. Percent survival after the feeding trial was also noted. The following formulas were used to compute growth parameters:

$$\text{SGR (\%)} = \frac{\ln(\text{final weight}) - \ln(\text{initial weight}) \times 100}{\text{days of feeding}}$$

$$\text{WG (\%)} = \frac{(\text{final weight}) - (\text{initial weight}) \times 100}{\text{initial weight}}$$

$$\text{FCR} = \frac{(\text{feed given in dry weight})}{\text{final weight} - \text{initial weight}}$$

$$\text{PER} = \frac{(\text{final weight}) - (\text{initial weight})}{\text{dry weight of protein in feed}}$$

$$\text{Survival (\%)} = \frac{(\text{initial no. of shrimp stocked}) - (\text{no. of mortalities}) \times 100}{\text{initial no. of shrimp stocked}}$$

Immunological assays

Hemolymph Extraction. Anticoagulant for hemolymph extraction was composed of shrimp salt solution (SSS) containing 450 mM NaCl, 10 mM KCl and 10 mM HEPES and added with 10 mM EDTA at pH 7.3 and 850 mOsm/ kg (Hernandez-Lopez *et al.*, 1996). Using a 1-ml syringe with 25 gauge needles washed with pre-cooled anticoagulant, hemolymph was drawn from the base of the pleopod at the first abdominal segment near the genital pore of the shrimp.

Hemolymph extracted from shrimp of the same treatment were pooled and used for the determination of total hemocyte count (THC), respiratory burst activity (RBA) and phenoloxidase (PO) activity. Seven to ten healthy shrimp were used for each immune index measured.

Total hemocyte count (THC). Hemolymph was transferred in a microcentrifuge tube containing a fixative (10% formalin in 0.45 M NaCl) and stored in 4 °C. A drop of Rose Bengal solution (1.2% Rose Bengal in 50% ethyl alcohol) was added to the fixed

sample and incubated for 20 min to allow staining. Using Neubauer's hemocytometer, hemocytes were counted and expressed as THC per ml of hemolymph (Joseph and Philip, 2007) using the following formula:

For corner block:

$$\text{cell count (cells/ml)} = \frac{\text{total no. of cells}}{(\text{no. of squares counted})(1 \times 10^{-4})}$$

For center block:

$$\text{cell count (cells/ml)} = \frac{\text{total no. of cells}}{(\text{no. of squares counted})(4 \times 10^{-6})}$$

Then, compute for THC using dilution correction factor (DCF):

$$\text{DCF} = \frac{\text{volume of hemolymph}}{(\text{volume of hemolymph}) + (\text{volume of anticoagulant})}$$

$$\text{THC (cells/ml)} = \text{DCF} \times \text{cell count}$$

Respiratory burst activity (RBA). Respiratory burst activity (RBA) of hemocytes was evaluated following the Muñoz *et al.* (2000) protocol with modifications. It was quantified using reduction of nitroblue tetrazolium (NBT) to blue formazan as a measure of superoxide anion (O_2^-) production.

In summary, 300 μl of anticoagulant-hemolymph mixture was loaded in each well of the microtiter plate and incubated for 2 hours at room temperature. Supernatant was removed and replaced with 50 μl of modified hank's balanced salt solution (MHBSS) medium and 50 μl of phorbol myristate acetate (PMA) working solution. 50 μl of NBT was added to each well and samples were incubated for 30 min. Supernatant was removed and hemocytes were fixed for 10 min by adding 200 μl absolute methanol. Fixed hemocytes were washed twice with 70% methanol which was then discarded and then air dried. Formazan deposits were solubilized in 120 μl 2M potassium hydroxide (KOH) and 140 μl dimethyl sulfoxide.

Absorbance of samples was read at 620 nm wavelength using ELISA reader (Dynamica LEDECT 96). 120 μl of KOH and 140 μl of DMSO served as blanks. Values were expressed as optical density (OD) per ml of hemolymph.

Phenoloxidase (PO) activity. Phenoloxidase activity was measured spectrophotometrically following the Hernandez-Lopez *et al.* (1996) procedure with slight modifications.

Anticoagulant-free hemolymph was collected and allowed to coagulate. To induce cell lysis and degranulation, samples were subjected to 4 freeze-thaw cycles, vortexed, and centrifuged at $7500 \times g$ for 15 min at 4°C . Supernatant (blood serum) was collected and 3 volumes of SSS buffer (SSS containing 100 mM CaCl_2) were added. 25 μl of sample was placed in each microtiter plate well. 25 μl of 0.1 % trypsin solution (1 mg trypsin dissolved in SSS buffer) was added and incubated for 30 min. 25 μl of 0.3 % L- DOPA(L-3,4- dihydroxyphenylalanine) was added. Samples were read immediately after color change set in.

Optical density of samples was measured at 492 nm using ELISA reader (Dynamica LEDECT 96). Absorbance values were recorded every minute for 4 min. PO activity was expressed as the change in absorbance (optical density) per min per volume of hemolymph. Enzyme activity is equivalent to 0.001 increase in absorbance (Joseph and Philip, 2007).

Viral Stock Preparation. WSSV was obtained from infected moribund shrimp from SEAFDEC, Tigbauan, Iloilo, Philippines. The viral stock solution was prepared following the method described by Chang *et al.* (2003). Gills, lymphoid organs, and attached epidermis of the WSSV-infected shrimp sufficient to make 30% biomass were removed, cut, and homogenized in 0.9% saline solution at 4 °C to make up a (1:9, w/v) viral stock solution. Supernatant was collected after centrifugation at 3000 x *g* for 10 min.

WSSV Challenge Procedure. After the 8-week feeding trial, experimental animals from the five treatment groups were subjected to WSSV challenge by immersion (Chotigeat *et al.*, 2004). An equal volume (10 ml) of viral stock solution was inoculated per 30-L of rearing water. This concentration was optimized to induce 100% mortality within 4 days in shrimp fed with T1 diet. The test animals were immersed in the infected water for 2 hours and then transferred to their corresponding treatment tanks stocked with fresh, UV-treated water with moderate aeration. The same feeding protocol in the growth trial was observed. The procedure was carried out in a static system. An unchallenged group served as negative control. Salinity was maintained at 20 ppt. and temperature at 27-28°C. Mortality was recorded daily until 100% mortality was observed in the control group. Dead and moribund shrimp were removed and subjected to polymerase chain reaction (PCR) analysis to confirm presence of WSSV. Electrophoresis of PCR products revealed a heavy band at 982 bp for one-step positive sample. Viral infection activity was performed in a biosecured facility.

Treatment of Wastewater. Wastewater was treated with 100 ppm sodium hypochlorite and held in a 300-L reservoir for 3 days before disposal. Tanks, equipment, and materials used during the challenge were soaked in 100 ppm sodium hypochlorite for 48 hours prior to rinsing and washing.

Statistical Analysis. One-way ANOVA, by SPSS 16.0 software, was used to check significant differences in the growth parameters and immunological indices of shrimp in the treatment groups at 0.05 level of significance. Results for WSSV test challenge were processed using randomized complete block design (RCBD). Post-hoc analysis using Duncan Multiple Range Test was done to determine the optimum feeding frequency of *V. harveyi* LPS that elicits best growth response, WSSV infection resistance, and immune responses i.e. THC, PO activity, and RBA in *P. monodon*.

Results

Growth performance. Growth parameters for *P. monodon* initially weighing 0.02 g, increased during the feeding trial. Final average body weight was recorded at 0.19-0.26 g. Average weight gain differed significantly between treatments (Fig. 1). Shrimp receiving test immunostimulant every 2 days elicited the best growth response which was significantly different from the control and the other treatment groups. Specific growth rate was similar (Fig. 2). No significant difference was observed in the feed conversion ratio (Fig. 3) and protein efficiency ratio (Fig. 4) of all test groups. Survival of shrimp after the 8-week feeding trial ranged from 90-96% with no significant difference between treatments (Fig. 5).

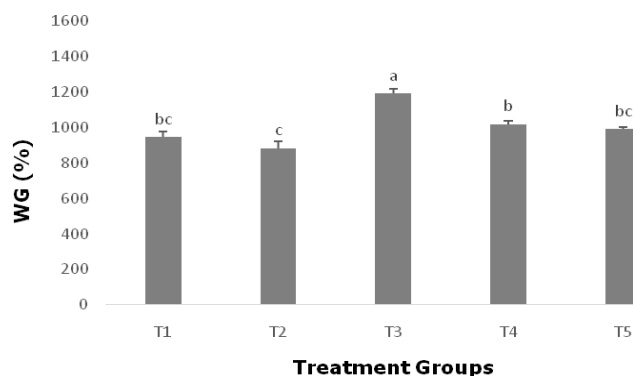


Fig 1. Cumulative weight gain (%) of *P. monodon* after 8-week feeding trial. Same superscripts indicate no significant difference ($\alpha = 0.05$). T1 – control, T2 – LPS fed daily, T3 – LPS fed every 2 days, T4 – LPS fed every 5 days, T5 – LPS fed every 7 days

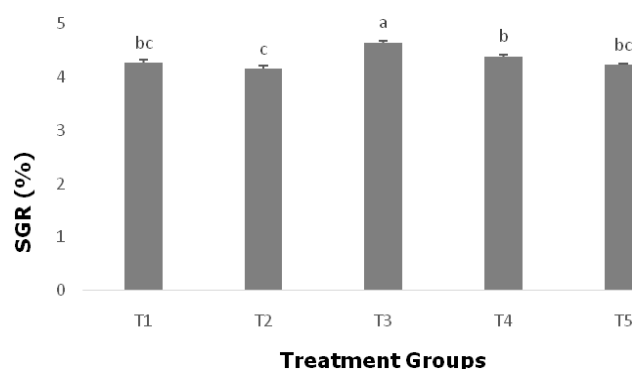


Fig 2. Specific growth rate (%) *P. monodon* after 8-week feeding trial. Same superscripts indicate no significant difference ($\alpha = 0.05$). T1 – control, T2 – LPS fed daily, T3 – LPS fed every 2 days, T4 – LPS fed every 5 days, T5 – LPS fed every 7 days

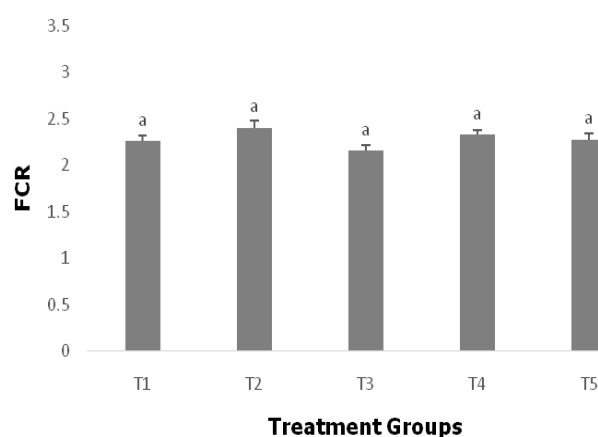


Fig 3. Feed conversion ratio of *P. monodon* after 8-week feeding trial. Same superscripts indicate no significant difference ($\alpha = 0.05$). T1 – control, T2 – LPS fed daily, T3 – LPS fed every 2 days, T4 – LPS fed every 5 days, T5 – LPS fed every 7 days

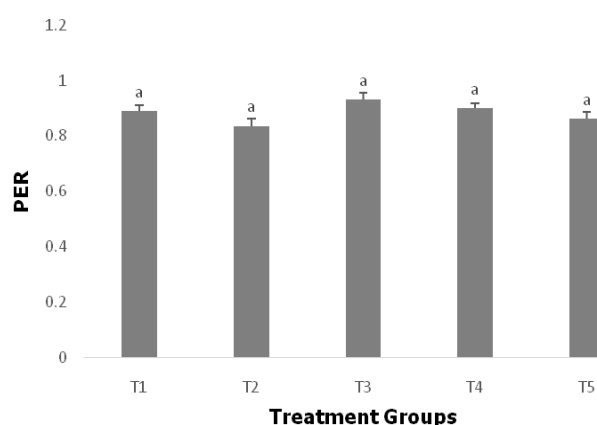


Figure 4. Protein efficiency ratio of *P. monodon* after 8-week feeding trial. Same superscripts indicate no significant difference ($\alpha = 0.05$). T1 – control, T2 – LPS fed daily, T3 – LPS fed every 2 days, T4 – LPS fed every 5 days, T5 – LPS fed every 7 days

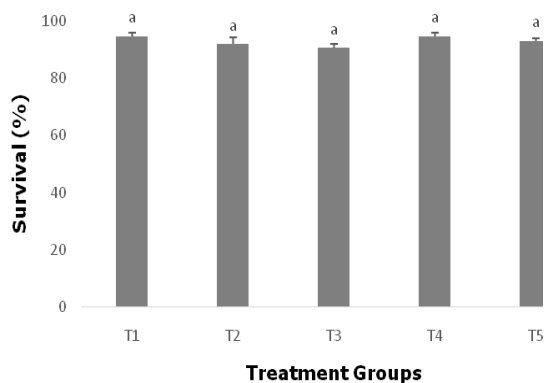


Figure 5. Survival (%) of *P. monodon* after 8-week feeding trial. Same superscripts indicate no significant difference ($\alpha = 0.05$). T1 – control, T2 – LPS fed daily, T3 – LPS fed every 2 days, T4 – LPS fed every 5 days, T5 – LPS fed every 7 days

WSSV challenge survival. Compared to all the other treatment groups including the control group, shrimp fed immunostimulant every 2 days were significantly resistant showing a 53% significantly higher survival rate at the end of the experiment (Fig. 6).

As expected, there was 100% mortality in the control on the 4th day while there was partial survival in the treated groups. Infected shrimp exhibited loss of appetite, lethargy, and white spots on the carapace. In the negative control group, no mortalities were recorded. All dead and moribund shrimp were analyzed by PCR and were found to be positive to WSSV by one-step amplification (982 bp) (Fig. 7), indicating that all mortalities were caused by WSSV infection.

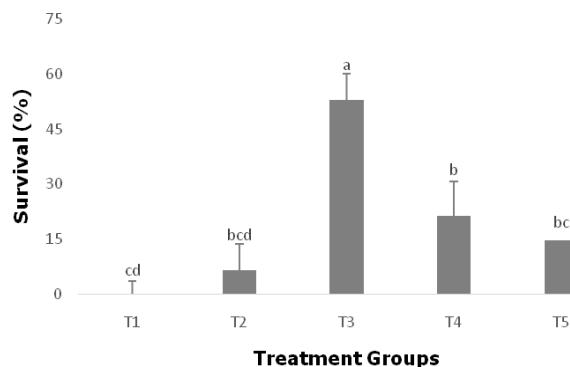


Figure 6. Survival (%) of *P. monodon* after WSSV test challenge. Same superscripts indicate no significant difference ($\alpha = 0.05$). T1 – control, T2 – LPS fed daily, T3 – LPS fed every 2 days, T4 – LPS fed every 5 days, T5 – LPS fed every 7 days

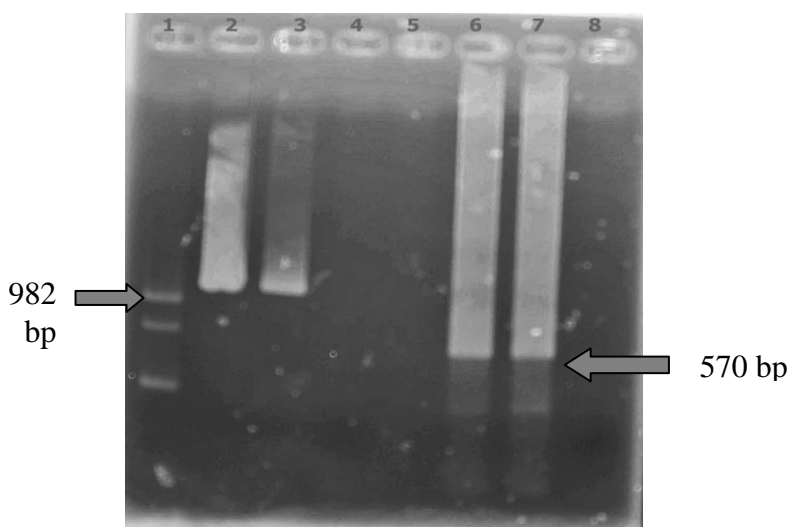


Figure 7. WSSV analysis by PCR. Lane 1 – 100-bp DNA marker (GeneReach Biotechnology), Lane 2 & 3 – one-step positive pooled samples, Lane 4 & 5 – SEAFDEC/AQD sample, Lane 6 & 7 – nested positive pooled samples. Expected products for one-step and nested positive are 982 bp and 570 bp, respectively.

Immunological assays

- *Total hemocyte count (THC)*

THC was 72% higher in the group fed the LPS-supplemented diet every 2 days than the control group, but was not significantly different from the group receiving LPS every 7 days (Fig. 8). Shrimp receiving immunostimulant every 5 days had the lowest cell count but did not differ statistically from the group fed LPS daily.

- *Phenoloxidase (PO) activity*

Shrimp receiving immunostimulant every 2 days and every 5 days exhibited 77% more enzymatic activity than the control group (Fig. 9). Significantly lower PO activity was observed in the group receiving the immunostimulant every 7 days with no significant difference from the untreated group.

- *Respiratory burst activity (RBA)*

Enhanced respiratory burst activity was recorded in the group receiving LPS every 7 days with no significant difference from the groups receiving LPS every 2 and 5 days (Fig. 10). On the other hand, O_2^- production was substantially suppressed in the control group.

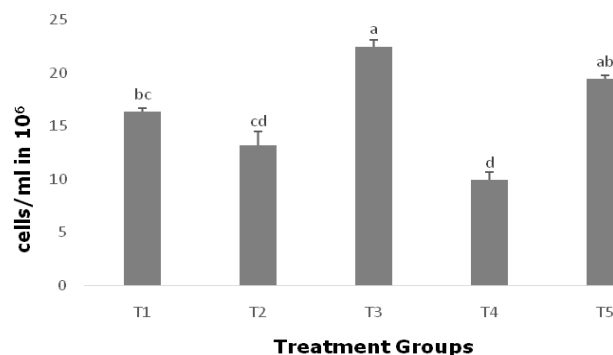


Figure. 8. Total hemocyte count (cells/ml x 10⁶) of *P. monodon*. Same superscripts indicate no significant difference ($\alpha = 0.05$). T1 – control, T2 – LPS fed daily, T3 – LPS fed every 2 days, T4 – LPS fed every 5 days, T5 – LPS fed every 7 days

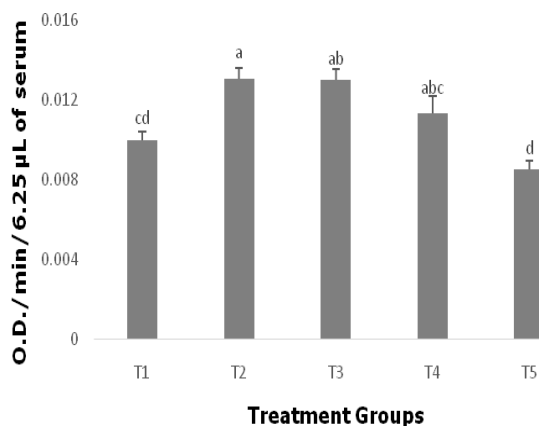


Figure 9. Phenoloxidase activity (O.D./min/6.25 µL of serum) of *P. monodon*. Same superscripts indicate no significant difference ($\alpha = 0.05$). T1 – control, T2 – LPS fed daily, T3 – LPS fed every 2 days, T4 – LPS fed every 5 days, T5 – LPS fed every 7 days

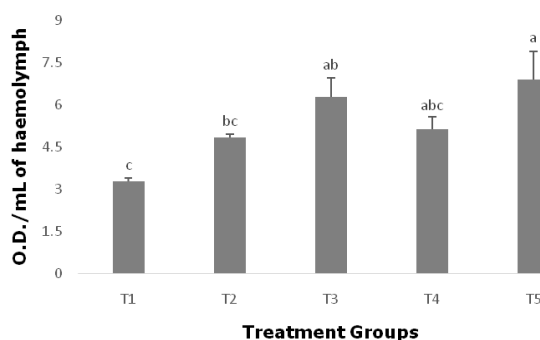


Fig. 10. Respiratory burst activity (O.D./mL of haemolymph) of *P. monodon*. Same superscripts indicate no significant difference ($\alpha = 0.05$). T1 – control, T2 – LPS fed daily, T3 – LPS fed every 2 days, T4 – LPS fed every 5 days, T5 – LPS fed every 7 days

Discussion

Several dietary immunostimulants of bacterial, fungal, and algal origins have been reported to promote better growth in crustaceans (Ganguly et al., 2010; Itami et al., 1998; Montero-Rocha et al., 2006; Traifalgar et al., 2009; Zhao et al., 2012). In the present study, the test immunostimulant exhibited a growth-enhancing effect on shrimp when given every 2 days. This was significantly different from the control and the rest of the treated groups. Similar enhancement effects on shrimp growth have also been observed in *P. japonicus* fed peptidoglycan-enriched diets in a long term feeding trial (Itami et al. 1998) and in *P. monodon* larvae fed diets with bacterin from *Vibrio vulnificus* (Song and Sung, 1990). The growth-promoting effect of LPS has been documented in several studies in finfish. Skalli et al. (2013) reported significantly enhanced body weight of juvenile rainbow trout when fed with LPS derived from *Pantoea agglomerans*. Biochemical body composition also revealed that LPS-fed fish displayed higher levels of crude protein which may account for the improved growth performance. Atlantic salmon fry receiving diets containing 0.01% and 0.03% *Aeromonas salmonicida* LPS showed better growth performance in comparison with fish fed a diet without LPS. (Guttvik et al. 2002). The positive effect of lipopolysaccharide on shrimp growth has not been widely documented. The mechanism by which it induces growth in test animals given optimum dose and frequency is yet to be investigated.

FCR and PER results proved that the inclusion of LPS in the diet did not inhibit the conversion of dietary protein into usable protein in shrimp. Survival of all treatment groups was high (90-96%) and not affected by supplementation of LPS.

Dose optimization studies on dietary LPS revealed that, beyond optimum dose, the protective effect of the test immunostimulant in shrimp against WSSV infection was not significant (Newman, 2000; Felix, 2005). Frequent and prolonged administration of immunostimulants, may in fact produce an overdose and may actually reduce their efficacy. Our study revealed that the group receiving the immunostimulant every 2 days showed significantly enhanced resistance against WSSV infection and had 53% survival at the end of the experiment while there was 100% mortality in the control group. The observed survival rate of shrimp receiving LPS every 2 days may be attributed to the protective effects of the compound due to the activation of several humoral and cellular immune responses in shrimp. Total hemocyte count, PO activity, and respiratory burst activity were markedly elevated. These three immune indices were indicators of the protective effect of LPS. A substantial drop in at least one of these indices resulted in decreased resistance of shrimp against the viral pathogen.

To the best of our knowledge, this paper is the first to investigate the effect of feeding frequency of LPS-supplemented diet at a predetermined dose of 50 mg/kg on the performance of post larval *P. monodon* against WSSV infection. Studies on the dose optimization of bacterial LPS on penaeids have been widely reported.

Despite enhanced levels of PO and superoxide anions, the group fed with dietary LPS daily performed poorly in the challenge test. This was most likely due to the extremely low hemocyte count seen in the results. This observation is similar to an *in vitro* study on *Litopenaeus vannamei* hemocytes which revealed a direct effect of high-dose LPS on the reduction of cell size and viability which appeared to be associated with degranulation of semi-granular cells (Xian et al., 2009). Excessive degranulation of circulating hemocytes overactivates prophenoloxidase cascade (Sajeevan et al., 2009). Overstimulation of the immune system when immunostimulants are given above the optimum dosage and frequency, causes a condition similar to "immune-fatigue" (Chang et al. 2003). This negative effect associated with frequent administration of immunostimulants is consistent with the results obtained by Flores-Miranda et al. (2011) who investigated the efficacy of commercial lactic acid bacteria and heat-killed yeast, in *L. vannamei* against *Vibrio sinaloensis* infection on a dose-frequency context. Results revealed that shrimp fed with immunostimulant-supplemented diet every 3 days had significantly higher survival than the group fed the immunostimulant daily. In the present study, there was not a strong correlation between frequency of administration of LPS and survival of the animals challenged by WSSV. While inclusion of LPS in the shrimp diet may activate innate immune responses, feeding at higher frequencies may induce toxicity. Unlike chemotherapeutics, immunostimulants do not seem to follow a linear dose/effect relationship (Bliznakov and Adler, 1972). In fact, there is an optimum level at

which they produce protective effects. Beyond this presumed optimum level, immunostimulants, at higher concentrations or prolonged exposure, have no effect or may in fact induce toxicity (Floch *et al.*, 1987). The importance of dose and frequency was demonstrated in the administration of glucan derived from *Candida sake* in the diet of *Fenneropenaeus indicus* post-larvae to act as an immunostimulant against WSSV infection (Sajeevan *et al.*, 2009). Optimum frequency of the test compound at a predetermined dose against WSSV was reported every 7 days (Sajeevan *et al.*, 2006). This was coupled with significantly elevated immune responses such as THC, PO activity, and RBA. It was presumed that at a higher dose, glucan may induce excessive degranulation of granular and semi-granular hemocytes, resulting in the overactivation of prophenoloxidase cascade causing immune fatigue in the shrimp. Immunosuppression most likely caused the rapid death of the experimental animals in the infection challenge. Using different yeast products as immunostimulants for *L. vannamei* in a long term feeding trial, growth and antibacterial clearance activities of *L. vannamei* fed immunostimulant-supplemented diet was significantly suppressed and the measured immune response was much lower than that of the control group (Scholz *et al.*, 1999). These results have become the subject for critique in a review paper challenging the reliability of immunostimulants in fish farming (Smith *et al.*, 2003). Generally, these studies imply that administration of immunostimulants beyond the presumed optimal level of dose, frequency, and length of feeding, may lead to overstimulation of innate shrimp functions possibly inhibiting immune defense activities that confer protection of organisms from certain diseases.

Each immunostimulant may activate different specific pathways in the crustacean immune system. Reported optimum dietary dose for LPS used in penaeid culture ranges from 20-100 mg/kg diet. These slight disparities can be attributed to the source of the tested immunostimulant, the phenological stages, and species of experimental animals. Nevertheless, survival rates against WSSV were similar in most studies. Growth and pathogenicity trials on the use of LPS in other crustaceans such as crabs are limited. These are particularly important for understanding host-specific immune defense activity activated by the test immunostimulant.

In summary, the results demonstrated positive effects of dietary supplementation of *V. harveyi* LPS on the growth and WSSV resistance of *P. monodon*. Effective immunostimulation was seen to increase in each of the three immune indices, total hemocyte count, phenoloxidase activity, and respiratory burst activity. Dietary *Vibrio harveyi* lipopolysaccharide fed every 2 days at a concentration of 50 mg/kg diet is optimum to promote growth, WSSV infection resistance, and immune responses in *P. monodon* post larvae.

The present findings demonstrate the importance of determining optimum application frequency of immunostimulants in shrimp culture. From a production point of view, the enhancement of growth, disease resistance, and immune responses, accompanied by a predetermined dose and frequency could be a useful approach towards increasing productivity and minimizing losses. Although the present work was conducted in laboratory conditions, the effectiveness of LPS has also been reported in some field-based experiments (Newman, 1999). In Thailand, when juvenile *P. monodon* were immersed and orally-treated with LPS there was a 25% increase in survival against WSSV in comparison with the untreated group. In a larger farm trial in Indonesia, shrimps bathed and subsequently fed with LPS were protected against WSSV, with only 4% of total number of ponds testing positive for WSSV. Considering its efficacy, inclusion of 50 mg LPS per kilogram of shrimp diet fed every 2 days is economically feasible on a commercial scale. Thus, the use of LPS could be an important part of health management in shrimp farming.

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